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CASE REPORT

Molecular detection of *Leptospira interrogans* in human tissues and environmental samples in a lethal case of leptospirosis

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Abstract A forensic case of suspected Leptospirosis with fatal course was resolved by the molecular detection of *Leptospira interrogans* in postmortem human tissues and in environmental samples. Polymerase chain reaction analysis and DNA sequencing confirmed the clinical diagnosis of Weil syndrome, and the death was considered to be an occupational accident with all the legal implications.

Keywords Leptospirosis · Postmortem diagnosis · Molecular biology · Postmortem sampling technique · Occupational death

Introduction

Leptospirosis is an infectious disease caused by pathogenic bacteria of the genus *Leptospira*. Transmission occurs by contact of abraded skin, conjunctiva, or mucous membranes with water contaminated by urine of infected rodents. Infection can occur after animal bites or, very rarely, between humans via infected blood or urine. Rodents are considered to be the reservoir of the infection. Leptospirosis is presumed to be one of the most widespread zoonosis, especially in tropical countries where it is endemic [1, 2]. Usually, in the developed world, deaths related to this illness are rare. Leptospirosis can cause a large spectrum of diseases in humans, from a subclinical infection (the majority of cases) to the Weil syndrome, an icteric form with a high mortality rate (5–15%) [3, 4]. Usually, the infection presents with headache, myalgia, abdominal pains, fever, and skin rash, but these symptoms can be very mild so that patients do not often seek medical care. Only 5–10% of the patients with leptospirosis present with the icteric form, often complicated by multiorgan involvement such as meningitis, acute renal failure, myocarditis and pulmonary symptoms (alveolar haemorrhage and acute respiratory distress syndrome) [5–10].

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Forensic case

A previously well 65-year-old man presented to the local emergency room with headache, myalgia, vomiting, abdominal pain and jaundice. He complained of drowsiness and vomiting over the last 6 days. At admission, he was conscious, jaundiced, afebrile, with a blood pressure of 120/80 mmHg, a respiratory rate of 18 per minute, the heart rate was 70 per minute, abdominal palpation was painful, peristalsis was absent, and the electrocardiogram was negative. Blood analysis revealed thrombocytopenia (19,000/mm^3), leukocytosis (from 10,900/mm^3 to 11,500/mm^3 in 2 h) with neutrophilia (97.1%), high levels of glucose (236 mg/dl), of urea (187 mg/dl), of serum creatinine (5.55 mg/dl), of serum bilirubin (16.87 mg/dl), of transaminase (AST 151 mU/ml and ALT 59 mU/ml), of amylase (351 U/l), of lipase (444 U/l). He was given intravenous penicillin G (3 mU every 4 h), octeotride, domperidone, and was rehydrated and catheter-

ised. The accurate history acquired from his wife revealed that he was a plumber, and 10 days before, he had worked in a neglected public toilet. At our request, the sanitary inspector examined the public toilet where the man had worked. Specimens of water, soil, rat faeces and the dead body of a rat were collected and submitted for microbiological analysis.

The autopsy

The external examination showed only mucosal and scleral jaundice and no signs of trauma. The autopsy revealed yellowish coloration of internal tissues and organs which were normal in size and weight. Tissue samples for histology and body fluids for toxicological analysis were collected. Tissue samples and body fluids were also collected using sterile instruments (blades, forceps and tubes) and stored at −70°C before microbiological analysis by molecular methods.

Microscopic examination showed mild oedema of the brain with perivascular haemorrhages, haemorrhages in the alveolar spaces, mild steatosis of the liver with centro-lobular ischemic areas and vasculitis (lymphocytic infiltrates of the vascular wall). Perl's stain was positive in the lungs, and Wathin–Starry stain for the detection of leptospires in the liver was negative. No alcohol was detected in the blood, and the toxicological analyses for drugs of abuse and therapeutic drugs in the urine were negative.

Microbiological analysis

The polymerase chain reaction (PCR) for detection of *Leptospira* DNA was performed on biological samples collected during the autopsy and on environmental samples. Human samples subjected to molecular analysis were lung tissue (1), liver (2), kidney (3), pancreas (4), brain (5), urine (6) and cerebrospinal fluid (7). Environmental samples were water (1), collected from several structures in the public toilet, soil (2), rat faeces (3) and tissues (4) obtained from a dead rat (Table 1).

DNA extraction and PCR All samples were homogenised, heat-inactivated at 95°C for 30 min and subjected to a DNA extraction procedure (High Pure PCR template preparation kit, Roche Diagnostics). To remove any PCR inhibitor, the DNA obtained from the rat and the environmental samples

Table 1 Detection of *Leptospira* DNA

Source	ID	Sample	PCR	Sequence
Human	Hum1	Lung	Positive	*L. interrogans*
	Hum2	Kidney	Positive	*L. interrogans*
	Hum3	Pancreas	Positive	*L. interrogans*
	Hum4	Liver	Positive	*L. interrogans*
	Hum5	Brain	Negative	
	Hum6	CSF	Negative	
	Hum7	Urine	Negative	
Environmental	Env1[a]	Water From Toilet 1	Negative	
	Env2	Water From Toilet 2	Positive	*L. interrogans*
	Env3	Water From Tap	Negative	
	Env4	Water From Toilet 4	Positive	*L. interrogans*
	Env5	Soil Outside The Pond	Positive	*L. interrogans*
	Env6	Soil Inside The Pond	Positive	*L. interrogans*
	Env7	Rat Faeces	Positive	*L. interrogans*
	Rat1[b]	Bowel	Negative	
	Rat2	Right Kidney	Negative	
	Rat3	Bladder	Positive	*L. interrogans*
	Rat4	Left Kidney	Negative	
	Rat5	Heart	Negative	
	Rat6	Lung	Negative	
	Rat7	Liver	Negative	
	Rat8	Brain	Negative	
Control Strains[c]	RGA	*L. interrogans*	Positive	*L. interrogans*
	Top1	*L. weilii*	Positive	*L. weilii*
	MoskV	*L. kirshneri*	Negative	

[a] Samples from Env1 to Env8 have been collected at the site of the accident.
[b] Samples from Rat1 to Rat8 came from the dissection of the rat.
[c] As expected, primers used for PCR analysis could detect *L. interrogans* and *L. weilii* DNA, but not *L. kirshneri* [11].

was dialysed by the Amicon Bioseparation (Millipore) system. The PCR assay was run essentially as described by Gravekamp et al. [11, 12], which detects a 285-bp region of the *secY* gene, typical for pathogenic *Leptospira* species. The PCR mix contained 2 μl of DNA samples, reaction buffer 1X, 0.8 U Taq polymerase (DyNazyme II DNA Polymerase, Fynnzymes Oy), 10 pmol of each primer, deoxynucleotide triphosphate 0.075 mM and water to a final volume of 25 μl. PCR conditions were as follows: one cycle at 95°C for 4 min, then 30 cycles at 50°C for 40 s, 72°C for 1 min and 92°C for 30 s. Amplification products were separated by gel electrophoresis on 1.5% agarose and observed under UV light after staining with ethidium bromide. As an amplification control, DNA from heat-inactivated cultures of *L. interrogans* and *L. weilii* were analyzed as positive controls and *L. kirshneri* as the negative control. Inactivated bacterial cultures were kindly supplied by Dr. Lorenzo Ciceroni [13] (Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy).

DNA sequencing PCR fragments from all positive samples were subjected to nucleotide sequencing. The direct automated sequencing of the purified PCR fragments was performed at Genelab (ENEA, Casaccia, Rome, Italy). Sequences of both DNA strands were determined, each using as template the product of a different PCR reaction. In the sequencing reaction, 6 pmol of the PCR primers G1 and G2 and 30 ng of the PCR product were employed. Analysis and comparison of sequence data were carried at the BLAST interface of the National Center for Biotechnology Information (http://www.

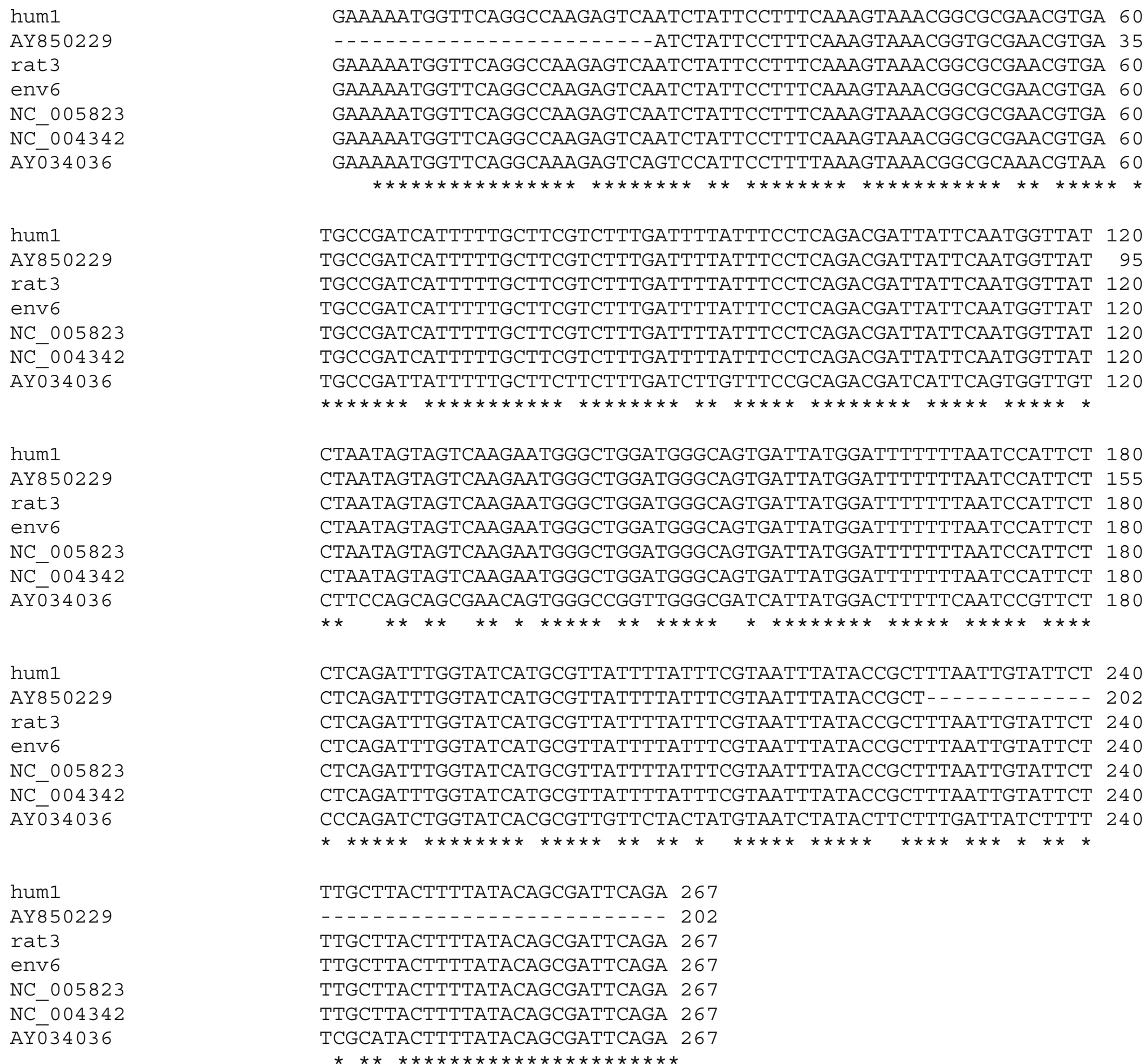

Fig. 1 Alignment of partial *secY* sequences. *L. interrogans* sequences obtained in this work from four human samples, five environmental samples and from a rat sample are identical and are represented in this alignment by sample hum1 (human lung sample), env6 (soil sample) and rat3 (rat bladder). Our sequences are identical to the *secY* sequences of *L. interrogans* serovar Copenhageni (NC_005823), Lai (NC_004342) and Baires (AY850229). Identity to the *secY* gene of *L. weilii* strain Ecochallenge is 82% (AY034036)

ncbi.nlm.nih.gov/blast/) and at the CLUSTALW interface of the European Molecular Biology Laboratory (http://www.ebi.ac.uk/clustalw/).

Results

Gel electrophoresis analysis of PCR products revealed a band of 285 bp, the expected size for *Leptospira* spp DNA, in four out seven human samples and in 6 out 15 environmental samples (Table 1). A positive reaction was obtained from human lungs, liver, pancreas and kidney. Environmental positive samples included water from two different toilets, soil from a pond (two samples), rat faeces and rat bladder. Sequence analysis of all the obtained PCR fragments identified the DNA as belonging to the bacterium *L. interrogans*. Comparison between sequences obtained from human and environmental samples and *L. interrogans* sequences deposited in GenBank showed all of them to be identical (Fig. 1), but distinct from *L. weilii*.

Conclusions

Molecular biology techniques are sensitive and specific tools for culture-independent diagnosis of microbial infections [14, 15]. In forensic pathology, a culture-based microbiological diagnosis is typically complicated by the frequent contamination of postmortem samples and by the possible lack of pathogen viability [12, 16]. Detection of microbial antigens and of microbe-specific antibodies may also be difficult due to postmortem alteration of antigens and antibodies [17–19]. Microbiological diagnosis on autoptic samples is best performed by molecular methods that allow detection of the pathogen nucleic acids. Advantages include (1) relative stability of the molecule to be detected (DNA), (2) detection of non-viable microorganisms and (3) contamination-independent detection of specific pathogens.

The case described here highlights the usefulness of molecular assays in postmortem diagnosis of microbial infections. Clinical presentation, laboratory data and autopsy results suggested leptospirosis; however, the final diagnosis was possible by DNA detection by PCR. In our protocol, diagnosis by standard PCR was confirmed by DNA sequencing of the amplicon, whereas by real-time PCR [20], diagnosis of leptospirosis is obtained by a single analytical procedure. *L. interrogans* DNA was detected in lungs, liver, kidneys and pancreas, unequivocally indicating the aetiology of the syndrome. *L. interrogans* DNA was also detected in environmental samples, indicating a high bacterial load and suggesting exposure to a high infectious dose. Concomitant detection of leptospiral DNA in human and environmental samples, in a non-endemic area [21], suggested that infection occurred as a consequence of working in a *Leptospira*-contaminated environment, and it could be classified as an occupational accident. This case underlines the importance of adequate sampling techniques [22–24] in forensic cases where a microbiological aetiology is suspected and a detection of microbial nucleic acids is foreseen.

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